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# Streamlined assessment of membrane permeability and its application to membrane engineering of Escherichia coli for octanoic acid tolerance

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## Abstract

The economic viability of bio-production processes is often limited by damage to the microbial cell membrane and thus there is a demand for strategies to increase the robustness of the cell membrane. Damage to the microbial membrane is also a common mode of action by antibiotics. Membrane-impermeable DNA-binding dyes are often used to assess membrane integrity in conjunction with flow cytometry. We demonstrate that in situ assessment of the membrane permeability of *E. coli* to SYTOX Green is consistent with flow cytometry, with the benefit of lower experimental intensity, lower cost, and no need for a priori selection of sampling times. This method is demonstrated by the characterization of four membrane engineering strategies (deletion of *aas*, deletion of *cfa*, increased expression of *cfa*, and deletion of *bhsA*) for their effect on octanoic acid tolerance, with the finding that deletion of *bhsA* increased tolerance and substantially decreased membrane leakage.



## Keywords

SYTOX, Membrane engineering, Octanoic acid, Lag phase, Cyclopropane fatty acids


## Disciplines

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## Comments

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# Streamlined assessment of membrane permeability and its application to membrane engineering of *Escherichia coli* for octanoic acid tolerance

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**ABSTRACT**

The economic viability of bio-production processes is often limited by damage to the microbial cell membrane and thus there is a demand for strategies to increase the robustness of the cell membrane. Damage to the microbial membrane is also a common mode of action by antibiotics. Membrane-impermeable DNA-binding dyes are often used to assess membrane integrity in conjunction with flow cytometry. We demonstrate that *in situ* assessment of the membrane permeability of *E. coli* to SYTOX Green is consistent with flow cytometry, with the benefit of lower experimental intensity, lower cost, and no need for *a priori* selection of sampling times. This method is demonstrated by the characterization of four membrane engineering strategies (deletion of *aas*, deletion of *cfa*, increased expression of *cfa*, and deletion of *bhsA*) for their effect on octanoic acid tolerance, with the finding that deletion of *bhsA* increased tolerance and substantially decreased membrane leakage.

**Keywords:** SYTOX; Membrane engineering; Octanoic acid; Lag phase; Cyclopropane fatty acids

## INTRODUCTION

A major hurdle to microbial production of biorenewable fuels and chemicals at economically viable titers, rates and yields is the fact that many of the target products, along with certain types of biomass-derived substrates, are harmful to the production organism [1-4]. One strategy for addressing this problem is to increase the robustness of the production organism to the problematic inhibitor, such as through evolutionary or targeted strain development, [3, 5-7]. In many cases, damage of the microbial cell membrane is a substantial component of the microbial inhibition, and thus the membrane is an attractive engineering target [8-17]. Engineering of the microbial cell membrane to combat this damage has been demonstrated, for example, to improve production of fatty acids [18-21], succinate [22], styrene [21] and hyaluronic acid [23].

One of the important metrics of membrane functionality is its permeability. Intact membranes are able to restrict the efflux of metabolically valuable molecules, such as ATP, while preventing the entry of potentially harmful molecules. Damage to the microbial cell membrane, such as by organic acids, alcohols, heat shock, and antibiotics, can be quantified by measurement of the membrane permeability either as leakage of molecules out of the cell [8, 9, 13, 24] or as the entry of molecules into the cell, such DNA-binding fluorescent dyes [16, 25-27]. There are a variety of DNA intercalating agents that only fluoresce when bound to double-stranded DNA [28]. Historically, DNA-binding dyes such as ethidium bromide and propidium iodide have been widely used, with dyes such as SYBR Green, Pico Green and SYTOX Green becoming more widely used in the last 25 years [29-31]. SYTOX Green has many desirable properties, including a relatively low rate of photobleaching and high specificity for DNA relative to RNA [31]. Most mechanistic studies of cell permeability use microscopic-based imaging of SYTOX permeability at the single-cell level [32, 33]. At larger scales, quantification of SYTOX Green permeability is typically performed by flow cytometry, with the sorting of cells as either SYTOX positive or SYTOX negative or a population distribution [16, 20, 25, 34-40]. A high-throughput assay using SYTOX Green to measure cell viability has been reported [41], though other reports have recommended that SYTOX Green should be used cautiously in the distinguishing of live and dead cells [42].

Given the increasing interest in quantification of membrane permeability, here we sought to streamline the assessment of engineering strategies for their effect on membrane integrity through the development of an *in situ* SYTOX assay. This high-throughput assay can be performed in conjunction with microbial growth with less required sample processing than flow cytometry, and with a promising economic assessment. The applicability of

this assay is demonstrated by the characterization of membrane engineering strategies aimed to increase tolerance of octanoic acid, a representative membrane-damaging biorenewable chemical.

## MATERIALS AND METHODS

Strains and culture conditions

All strains are derivatives of *E. coli* MG1655, engineered through FLP/FRT method [43] (Table 1) using the primers listed in Table S1. Cells were grown at 37ºC in Luria Broth or MOPS defined minimal medium [44] with 2.0% w/v dextrose, both with an adjusted initial pH of 7.0. Chloramphenicol and ampicillin were added to final concentrations of 35 and 100 mg/L as needed.

**Table 1**. Strains utilized in this study.

| Strains | Genotype | Reference |
|---|---|---|
| MG1655 (WT) | F- lambda- *ilvG- rfb*-50 *rph*-1 | Jarboe's Lab |
| control | MG1655, Δ*ldhA*::FRT-*cat*-FRT, $Cm^+$ | [21] |
| +*cfa* | MG1655, pCA-*cfa*, $Amp^+$ | [45] |
| Δ*cfa* | MG1655, Δ*cfa*::FRT-*cat*-FRT, $Cm^+$ | [45] |
| Δ*bhsA* | MG1655, Δ*bhsA*::FRT-*cat*-FRT, $Cm^+$ | This study |
| Δ*aas* | MG1655, Δ*aas*::FRT-*cat*-FRT, $Cm^+$ | This study |

Measurement of membrane permeability

SYTOX Green (Thermo Fisher, Rockford IL) was provided from a 5.0 mM stock solution in DMSO and, unless stated otherwise, was added to a final concentration of 5.0 μM. For flow cytometry, 500 μL of cell culture were washed once with 1 mL of PBS (pH 7.00 ± 0.02) and resuspended to an $OD_{550}$ of 0.1 in PBS with 5.0 μM SYTOX Green. These cells were grown in 96-well plates with a total culture volume of 200 μL, with samples from multiple wells combined for a total volume of 500 μL. Flow cytometry was performed at the ISU Flow Cytometry Facility with a BD Biosciences FACSCanto flow cytometer, as previously described [46]. For the *in situ* assessment described here, 200 μL of cell culture containing 5.0 μM SYTOX Green was loaded into a flat-dark bottom 96-well plate with total well volume of 373 μl (Thermo Scientific, Rockford IL) to track bulk fluorescence. Bulk fluorescence was quantified in a SynergyHT BioTek microplate reader utilizing a filter of 485/20 nm and 516/20 nm for excitation and emission wavelengths. The samples were excited from the top and the normalized bulk fluorescence was calculated by dividing the bulk fluorescence by the $OD_{550}$ at the same time point.

Isopropanol challenge and thermal stress

*E. coli* MG1655 was grown overnight in 50 ml MOPS containing 2.0% w/v dextrose at pH 7.0 in 250 ml baffled flasks at 37 °C and 200 rpm. The culture was washed once with a similar volume of PBS (pH 7.00 ± 0.02) and diluted in PBS to $OD_{550}$ = 2.0 ± 0.2. One mL of diluted cells were challenged with either isopropanol or exposure to boiling water. For isopropanol challenge, PBS containing various concentrations of isopropanol was added and the mixture held at room temperature for 1 h. For thermal stress, cells were submerged in boiling water for up to 5 min. Membrane permeability of cells after treatment was assessed as described above.

Strain characterization during C8 challenge

A single colony was grown in 2 mL LB for 4 h at 37 ºC 250 rpm. Sixty μL of these log-phase cultures were centrifuged at 6000 RPM for 6 min and cell pellets were resuspended in 3 mL MOPS 2% w/v dextrose at pH 7.0. These seed cultures were grown overnight (~19 h) at 37 ºC 250 rpm with chloramphenicol. One hundred and fifty μL of seed culture were centrifuged at 6,000 rpm for 6 min and resuspended in 1.0 mL of MOPS 2.0% w/v dextrose, in some cases containing 10 mM octanoic acid, pH 7.00 ± 0.02. Two hundred μL of this cell culture was loaded into a clear-bottom 96-well plate to track $OD_{550}$, with another 200 μL of this culture being used for the *in situ* SYTOX assessment. The $OD_{550}$ was measured in an Eon BioTek microplate reader maintained at 37 ºC and 205 cpm. Specific growth rates were calculated during the log-phase by calculating the slope of the linearized growth rate equation $\ln (OD/OD_0) = \mu t$.

Statistics

Data Analysis of Microsoft Office Excel was utilized for testing significance of data. T-test values had a confidence interval of 95%. Regression and ANOVA tools were performed on data comparing toxicity of SYTOX Green. ANOVA single factor and Tukey test were performed on data analyzing specific growth rates of the different *E. coli* strains.

## RESULTS AND DISCUSSION

The increasing interest in the microbial cell membrane as an engineering target increases the need for accessible membrane characterization techniques. The commonly used method of assessing membrane integrity via

the SYTOX Green nucleic acid dye involves cell assessment by flow cytometry [25]. Such analysis requires *a priori* selection of sampling time and an experimental cost which often scales according to the number of experimental data points. Here, we investigate the modification of the typical SYTOX membrane permeability assessment to an *in situ*, real-time format, with an anticipated lower cost (Fig. 1A).

**SYTOX is suitable for *in situ* microbial characterization**

The sensitivity and range of the SYTOX nucleic acid binding is demonstrated in Fig 1B. Specifically, purified DNA was incubated with 5 μM SYTOX in PBS at pH 7.0 and the bulk fluorescence measured in a 96-well plate (Fig. 1B). A linear response curve across two orders of magnitude of DNA concentration, from 0.5 ng/μL – 125 ng/μL, was observed. Using estimated limits of 7.6 – 19.4 μg DNA/$10^9$ cells [47] and 6 – 23x$10^8$ cells/mL/OD [48], this DNA concentration range corresponds to OD values of roughly 0.1 – 2.5, within the range of standard OD values used in microbial growth experiments. Experiments performed with lower and higher DNA concentrations were outside the linear range of the plate reader (*data not shown*).

The concentration of 5 μM SYTOX was validated as saturating in the presence of 100 ng/μL DNA (Fig 1C), corresponding to a microbial OD of approximately 2. Though the DNA-binding associated with nucleic acid dyes is known to be mutagenic in some contexts [49], cells grown in the presence of 5 μM SYTOX showed no significant difference in specific growth rate relative to the no-SYTOX control ($p = 0.6$). However, the growth rate was shown to decrease in the presence of higher SYTOX concentrations (Fig. 1D, $p = 0.01$).

**SYTOX measurement *in situ* is consistent with flow cytometry**

The use of flow cytometry in the SYTOX assay was previously validated in comparison to microscopic imaging using cells damaged either by exogenous isopropanol or by submersion in boiling water [25]. The *in situ* SYTOX assay proposed here was similarly validated side-by-side with flow cytometry-based assessment (Fig. 2). Specifically, both methods measured an increase in cell permeability to SYTOX in response to increasing concentrations of isopropanol and to increased exposure time to high temperatures (Fig. 2A, 2B). A high degree of correlation was observed for the two assessment methods ($R^2 = 0.96$) (Fig 2C), validating the use of the *in situ* assay as an alternative to assessment by flow cytometry.

These experiments used stationary phase cells diluted to an OD of approximately 2.0. Stationary phase *E. coli* cells have previously been described as containing, on average, four genome equivalents [50], leading to an estimate of 19 μg DNA/$10^9$ cells. In conjunction with an estimate of $22x10^8$ cells/mL/OD for stationary phase cells [48], a bulk DNA concentration of 83.6 μg/mL is expected at OD 2.0. This concentration of DNA is expected to give a maximum bulk SYTOX signal of approximately 3,000, if all DNA is available for SYTOX binding (Fig. 1B). However, the maximum SYTOX signals observed were approximately 2,000 for the isopropanol challenge and 1,600 for thermal stress, suggesting that perhaps not all DNA was available for SYTOX binding.

***in situ* characterization can account for changes in cell density**

While the comparison of *in situ* and flow cytometric assessment described above is promising, these experiments did not involve microbial growth. The ability to characterize metabolically active cells is critical to determination of microbial robustness and the effectiveness of membrane engineering strategies. As a proxy for microbial growth, the challenge with exogenous 10% v/v isopropanol was repeated using a range of cell densities (Fig. 3A). As expected, the measured SYTOX signal for the *in situ* assay increased linearly in response to increasing cell concentration, due to the associated increase in available DNA. When these bulk fluorescence values were normalized by cell $OD_{550}$, the resulting values were constant across all cell concentrations. These results suggest that changes in cell concentration during the course of an *in situ* assessment can be accommodated by normalization with the cell density.

This approach of normalizing *in situ* fluorescence measurement by cell density was then applied to a typical growth curve-type experiment. Specifically, an overnight culture of control strain *E. coli* (MG1655 Δ*ldhA*) was inoculated into MOPS glucose defined media and split into two parallel experimental tracks. Part of this culture was placed in a clear-bottom 96-well plate for real-time measurement of $OD_{550}$, and the other was placed in a black-bottom 96-well plate with 5 μM SYTOX for real-time measurement of fluorescence. These two plates were incubated in separate plate readers, both of which were maintained at 37 °C and 205 cpm.

The growth measurements are as expected, with the typical lag, exponential and stationary phases apparent (Fig 3B). The bulk fluorescence values follow a similar trend, increasing over time and then leveling off as the cells entered stationary phase (Fig 3B). Normalization of the fluorescence measurements by the measured $OD_{550}$ at the corresponding time point (Fig 3C) shows an initial spike in membrane permeability during the first two hours, with

values leveling off for the rest of the experiment. This transient spike in membrane permeability is consistent with reports of transient increases in the intracellular concentration of metals during lag phase, as well as substantially increased susceptibility to hydrogen peroxide [51].

This repeated measurement of fluorescence over time does raise concerns about photobleaching of the SYTOX dye. Previous comparison of nine different nucleic acid dyes ranked the bleaching speed of SYTOX in the lowest third, with approximately 60 rounds of scanning at 488 nm required for a 50% decrease in signal intensity [31]. Here, we have measured the fluorescence intensity every 20 minutes for 15 hrs, thus nearing the 60 rounds of scanning associated with 50% decrease in signal. However, photobleaching of a fluorescent reporter requires that the molecule be excited at the time of the scan [52], and thus photobleaching should be less of a concern over periods in which the amount of exposed DNA available for SYTOX binding is low. The fact that the bulk SYTOX intensity was above 4,000 even after 10 hours of assessment (Fig 3B), a value that is consistent with the maximum observed intensity of 5,000 units observed in the experiments performed with free DNA (Fig 1) indicates that substantial photobleaching has not occurred. However, the raw fluorescence signal did decrease over the last 5 hours of measurement, with increasing variability between technical replicates, possibly indicating photobleaching.

### *in situ* assessment of membrane engineering strategies

The applicability of this assay is further demonstrated through the characterization of four membrane engineering strategies that aim to increase tolerance to exogenous octanoic acid (C8). The damaging effects of carboxylic acids on the integrity of microbial cell membrane have been previously and extensively described [9, 14-16, 53]. The *in situ* SYTOX assay described here allows monitoring of changes in the membrane permeability in real time without *a priori* selection of sampling times (Fig 4).

For the wild-type strain, the presence of 10 mM exogenous C8 during growth in glucose minimal medium decreased the specific growth rate from 0.49 to 0.20 $h^{-1}$, consistent with previous reports (Fig. 4A). However, the *in situ* characterization of the membrane permeability performed here suggests that C8 only decreases membrane integrity relative to the untreated control in late log (Fig. 4B). Given that previous reports of membrane integrity during exogenous fatty acid challenge involved only a single time point [9, 16], these results provide novel insight into the effect of C8 on the *E. coli* membrane.

It was previously reported that deletion of acyltransferase/acyl-ACP synthetase (*aas*) improved tolerance and production of fatty acids by preventing the incorporation of medium-chain length fatty acid tails into the cell membrane [18]. We implemented this engineering strategy in MG1655 and characterized the growth and membrane permeability of the resulting strain during challenge with 10 mM exogenous C8 in glucose minimal medium at pH 7.0 (Fig. 4). Unlike the previous report, we observed a significant ($p < 0.05$) decrease in specific growth rate during C8 challenge for the Δ*aas* strain relative to the control strain (Fig. 4A). This difference in outcomes could be due to the fact that the previous reports used rich media containing glycerol [18], while our characterization was performed in glucose mineral salt medium, where these two conditions have previously been shown to be associated with differential impacts of membrane engineering strategies related to fatty acid tolerance [19]. The *in situ* SYTOX assessment during C8 challenge indicates that the Δ*aas* strain has a membrane permeability that is consistently higher than the control strain (Fig 4B). The *in situ* characterization strategy allows observation of this difference across the entire experiment. The lower membrane integrity in the *Δaas* strain could possibly explain the lower specific growth rate.

Another previously-described membrane engineering strategy is tuning of the abundance of cyclopropane fatty acids via modulation of the expression of *cfa*. While the abundance cyclopropane fatty acid lipid tails has been shown to relate to tolerance of acid, heat and pressure [54, 55], limited success has been achieved in using these as a strategy for increasing tolerance of membrane-damaging bio-products such as carboxylic acids or alcohols [8, 9, 56]. Here, we performed *in situ* characterization of two strains previously engineered for altered cyclopropane fatty acid content [45]. The Δ*cfa* strain was previously shown to produce no cyclopropane fatty acids [45] and here showed a significant ($p < 0.05$), but small, increase in specific growth rate relative to the wild-type control (Fig. 4A). The strain engineered for increased cyclopropane fatty acid content expression of *cfa* from a plasmid was previously shown to have a higher abundance of cyclopropane fatty acids during challenge with exogenous C8 [45] and here showed no difference in specific growth rate relative to the control. Previous assessment of these strains by flow cytometry at a single time point concluded that there was no difference in membrane permeability during C8 challenge relative to the wild-type control [45]. The *in situ* assessment of membrane permeability for these strains described here allows assessment of the membrane permeability for the entire duration of the experiment, relative to a few discrete time points. This characterization indicates that the membrane permeability of the Δ*cfa* strain closely mirrors the control strain until during early, mid and late log phase and that the +*cfa* strain, while showing the same

trends as the control strain, has a consistently higher membrane permeability at all time points (Fig. 4B). Thus, the *in situ* characterization provides additional insight relative to sampling for flow cytometry at only a single or a few time points.

Finally, we characterized the effect of deletion of multiple stress resistance outer membrane protein BhsA. Perturbation of the abundance of this gene previously showed promise as a membrane engineering target in terms of its effect on cell surface hydrophobicity and fatty acid production titers [57]. Specifically, it has been reported that cells with increased surface hydrophobicity via plasmid-based expression of *bhsA* showed a corresponding increase in fatty acid production titers. Here, we performed *in situ* characterization of the growth and membrane permeability of *ΔbhsA* strain (Fig. 4). This strain showed a significant, though small, increase in specific growth rate relative to the control strain during C8 challenge, but the difference in membrane permeability between these two strains was dramatic (Fig 4B). The membrane integrity of the Δ*bhsA* strain was higher than the control strain during early and mid-log growth and did not show the late log increase in permeability displayed by all of the other strains characterized here during C8 challenge. This provides further support that *bhsA* is an interesting target for modifying relevant membrane properties such as hydrophobicity and permeability, which also impact features of interest such as fatty acid productivity and tolerance respectively.

Side-by-side comparison of the *in situ* SYTOX measurement to measurement via flow cytometry show similar trends (Fig 4C, 4D). Specifically, both methods indicate that the Δ*aas* strain and the *ΔbhsA* strain have increased and decreased, respectively, growth and membrane permeability relative to the control strain. These results provide further demonstration that *in situ* characterization of SYTOX permeability can provide data that is qualitatively similar to flow cytometry, with the benefit of not requiring *a priori* selection of sampling times. An interesting feature that might be altered by both genetic changes and the presence of inhibitors is cell morphology. These potential morphological changes are not captured by the *in situ* assessment for measuring membrane permeability described here.

### *in situ* analysis is promising on both a cost and time basis

Thus far we have demonstrated the suitability of this *in situ* SYTOX assay for standard microbial characterization experiments, in terms of its consistency with standard flow cytometric analysis. This assay also has the benefit of requiring less sample handling and not requiring *a priori* selection of sampling times. Finally, we

present an analysis of the cost of this method relative to flow cytometry both in terms of reagent cost and equipment usage fees.

A typical cost for SYTOX Green is $237 for 250 μL of a 5 mM stock, corresponding to a cost of $0.19/nmol. The *in situ* assay described here was performed in a 96-well plate with a culture volume of 200 μL. Dosing of a single well with 5 μM SYTOX Green would require 1 nmol of SYTOX Green ($0.19). This single culture can be monitored over time, with no additional cost incurred per time point, other than the associated equipment maintenance and bulb use. The device used here has a stated average lamp life of 1,000 hrs with a replacement cost of less than $150.

**Table 2**. Shared equipment user fees from five randomly selected US facilities.

| Facility | Flow cytometry cost ($/hr) | | Plate reader cost ($/hr) | |
|---|---|---|---|---|
| | internal user | external user | internal user | external user |
| 1 | 52 | 135 | 28 | 50 |
| 2 | 162 | 162 | 16 | 32 |
| 3 | 70 | 110 | 40 | 50 |
| 4 | 65 | 130 | 96 | 151 |
| 5 | 54 | 203 | 0 | 33 |
| Average cost | 81 | 148 | 36 | 63 |

Contrastingly, sampling of this single culture for analysis by flow cytometry typically requires a volume greater than 200 μL. For example, the addition of 5 μM SYTOX to 1.00 mL of cell culture would require 5 nmol of SYTOX ($0.95), with the total amount of SYTOX required and sample preparation and processing efforts scaling linearly with the number of time points.

Many research institutions provide shared access to the equipment types used here and charge an hourly rate to users, where this rate usually varies for internal vs external users (Table 2). Random sampling of five US facilities show average internal user fees of $81/hr for flow cytometry and $36/hr for plate reader usage. In an ideal scenario, approximately 60 samples could be processed in one hour of flow cytometer usage. However, in many cases users a charged a minimum amount of machine time, even when processing only a few samples. For the experiments described here, a user would need to use one plate reader for monitoring bulk SYTOX fluorescence and a second plate reader for monitoring cell growth in parallel.

Consider an 8-hr experiment with 60 distinct microbial cultures, accounting for different strains and replicates. Performing *in situ* characterization of these 60 samples in a 96-well plate with a culture volume of 200

μL would require 60 nmol of SYTOX ($11) and $576 in total user fees for two plate readers. Standard assessment of these 60 samples by flow cytometry, with sampling in 1-hour intervals, would require 2.4 μmol of SYTOX ($456), plus the total associated user fee of $528. Thus, the *in situ* assessment would cost $588 with essentially no required sample processing during the course of the experiment and permeability could be assessed at even shorter intervals, such as 20 minutes. The hourly assessment by flow cytometry would cost $984 and would require extensive sample handling. It should be noted, of course, that the specifics of the economic trade off will vary according to institution and equipment availability. This analysis indicates that, on average, the *in situ* assessment is preferable in terms of reagent cost, user fees and required sample processing.

## CONCLUSIONS

Here, we have demonstrated a modification of the typical SYTOX Green-based assessment of membrane permeability to streamline and economize first-pass characterization of engineered strains. This assay can be performed over the entire growth period without requirement of *a priori* selection of sampling times. This method is unlikely to detect changes in cell morphology, where such changes may impact the normalization of the bulk fluorescence value when comparing strains and conditions. It is recommended that all key findings be verified through traditional flow cytometric assessment or other techniques.

Theoretically, this approach is expected to work with other cell types. SYTOX Green has previously been used to characterize not just membrane permeability of *E. coli*, but also *Bacillus* species [32, 33], *Saccharomyces cerevisiae* [39, 40], *Candida albicans* [38], *Fusarium graminearum* [38], Drosophila neuronal cells [36], and human breast cancer cells [37]. This approach can possibly also be extended to other DNA-binding dyes used to assess the status of the microbial cell membrane, such as propidium iodide [58].

A trade-off of the *in situ* characterization relative to flow cytometry is the absence of a measurement of the population distribution, where such information could be relevant to measurement of population heterogeneity or the characterization of persister cells. Photobleaching may also be a concern and it is suggested that key results be confirmed using standard methods.

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**Figure Legends**:

**Figure 1: Bulk fluorescence is a good indicator for quantifying exposed DNA.**

(A) Membrane leakage can be approximated based solely on the binding of SYTOX Green to exposed DNA inside cells with lower membrane integrity with quantitative results comparable to assessment by flow cytometry. (B) Bulk SYTOX Green fluorescence in response to binding to purified DNA in PBS at room temperature, pH 7.00 ± 0.02 across two orders of magnitude. The plate reader utilized a filter for 485/20 nm and 516/20 nm for excitation and emission wavelengths respectively. The coefficient of variation (CV) ranged from 0.6 to 10% for the three replicates of each DNA concentration. (C) The standard concentration of 5µM SYTOX Green is sufficient to bind the highest DNA concentration (125 ng/µL) within the linear range of the plate reader. The CV was less than 2.5% for the three replicates of each SYTOX Green concentration. (D) The presence of 5 µM SYTOX Green did not significantly affect the log-phase specific growth rate in MOPS 2% w/v dextrose at 37 °C in 200 µL cultures of *E. coli* MG1655, though higher concentrations did appear to negatively impact growth.

**Figure 2: Measurement of bulk fluorescence positively correlates with assessment by flow cytometry.**

*E. coli* MG1655 was grown in MOPS 2.0% w/v dextrose medium at 37 ºC overnight, washed once with PBS (pH 7.00 ± 0.02) and diluted to $OD_{550}$ = 2.0 ± 0.2 in either (A) PBS containing isopropanol, and held at room temperature for 1 h or (B) submerged in water at 100°C. The CV was less than 2% for the three replicates of each condition. Independent samples were taken for flow cytometry and plate reader analyses, both using 5 µM SYTOX Green (C). The two methods give highly similar results. The correlation coefficient was statistically significant ($p < 0.0001$).

All values are the average of three replicates with error bars indicating the standard deviation.

**Figure 3: Normalized bulk fluorescence enables the quantification of membrane leakage in growing cultures.**

A) *E. coli* MG1655 was challenged with 10% (v/v) isopropanol for 1 hr at room temperature at various $OD_{550}$ values. Bulk fluorescence (white circles) was assessed using a plate reader. Bulk fluorescence values were normalized (black circles) relative to the cell optical density at 550 nm. The CV was less than 5% for the three replicates of each measurement. (B) *E. coli* (MG1655 Δ*ldhA*) was grown in MOPS 2.0% w/v dextrose medium with chloramphenicol at pH 7.0 for 15 h and 205 cpm (cycles per minute) in parallel in two distinct plate readers. One

device measured $OD_{550}$ and the other measured bulk fluorescence from 5 μM SYTOX, each measuring every 20 minutes. (C) The bulk fluorescence values were normalized by the corresponding $OD_{550}$ value.
All values are the average of three replicates with error bars indicating the standard deviation.

**Figure 4. *in situ* assessment of membrane engineering strategies to increase C8 tolerance**

All strains were characterized during growth in MOPS 2.0% w/v dextrose containing 10 mM C8 at 37°C with an initial pH of 7.00. Characterization of (A) growth and (B) *in situ* membrane permeability identify deletion of *bhsA* as a promising membrane engineering strategy for increasing growth and decreasing membrane damage in the presence of exogenous C8. The CV was less than 10% for the three replicates of each measurement. The inset of (A) shows specific growth rates, with letters indicating statistically significant ($p < 0.05$) groupings. Comparison of the log-phase specific growth rate and membrane characterization at the 6 hr time point via (C) *in situ* fluorescence measurements or (D) flow cytometry give qualitatively similar results.

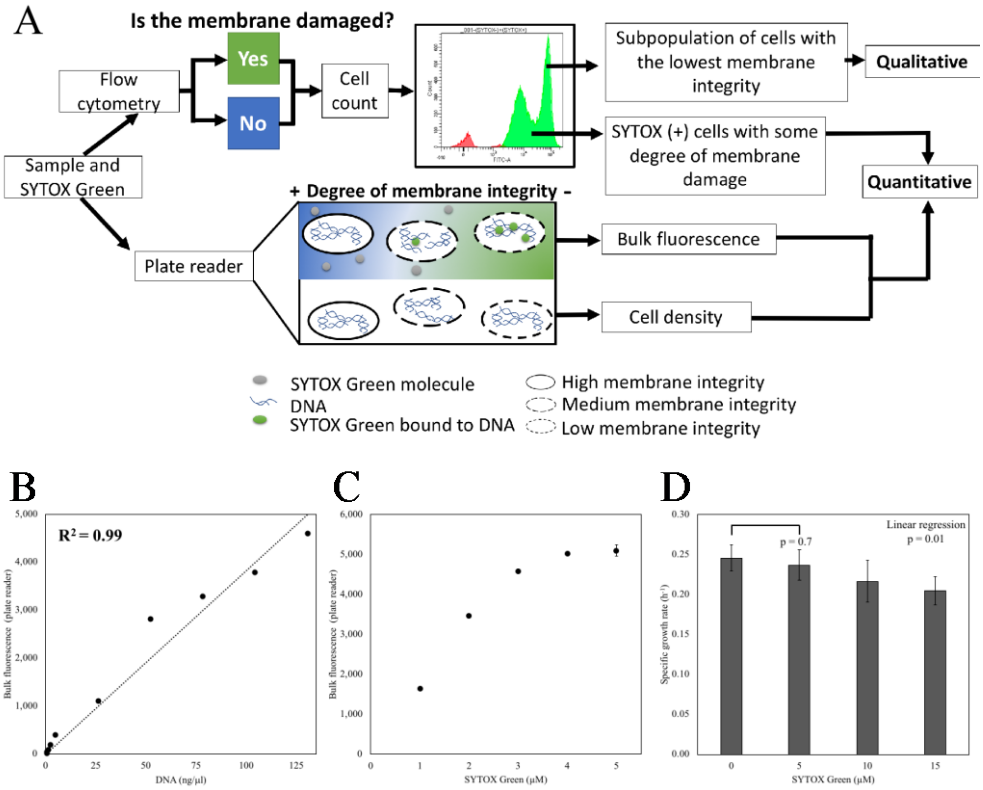


**Figure 1: Bulk fluorescence is a good indicator for quantifying exposed DNA.**

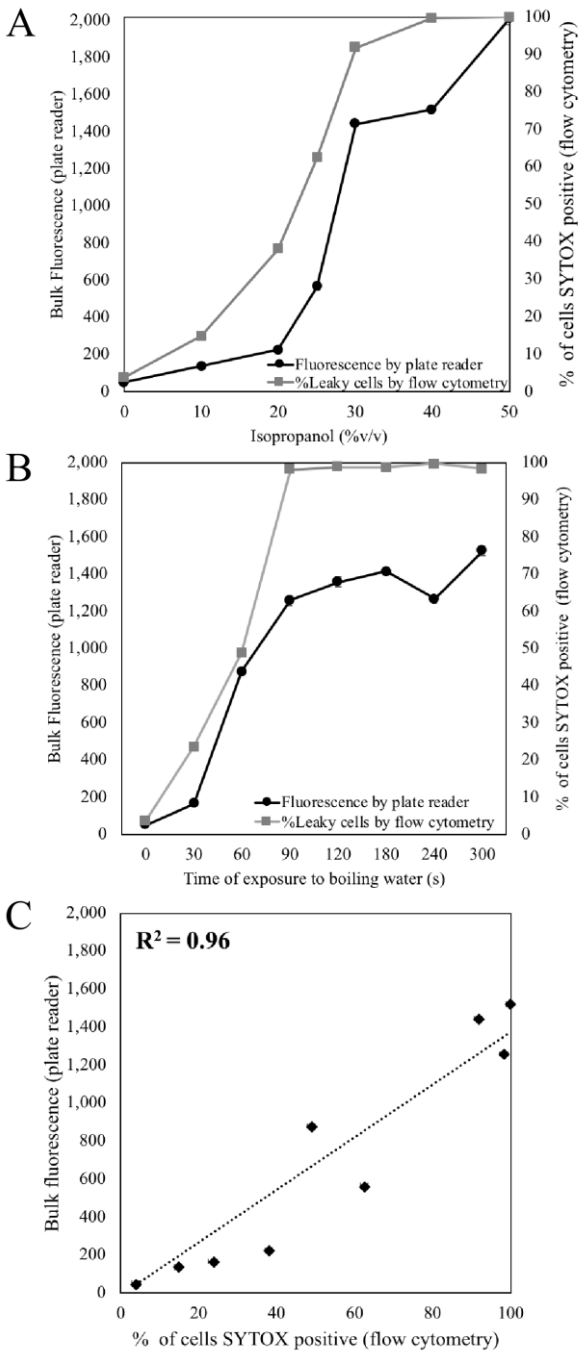


**Figure 2: Measurement of bulk fluorescence positively correlates with assessment by flow cytometry.**

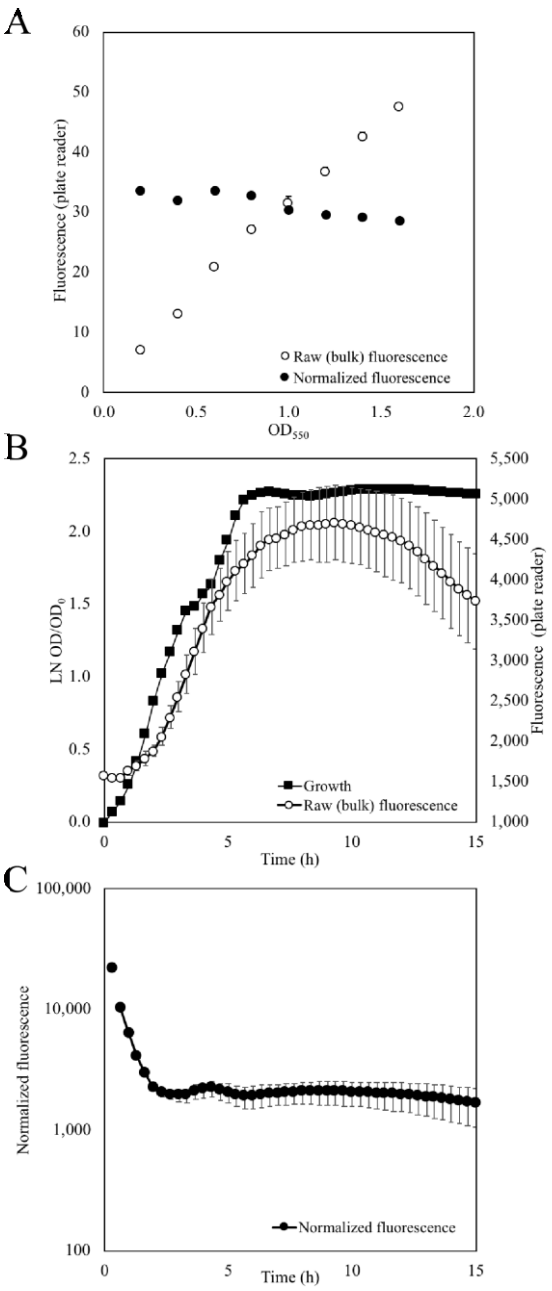


**Figure 3: Normalized bulk fluorescence enables the quantification of membrane leakage in growing cultures.**

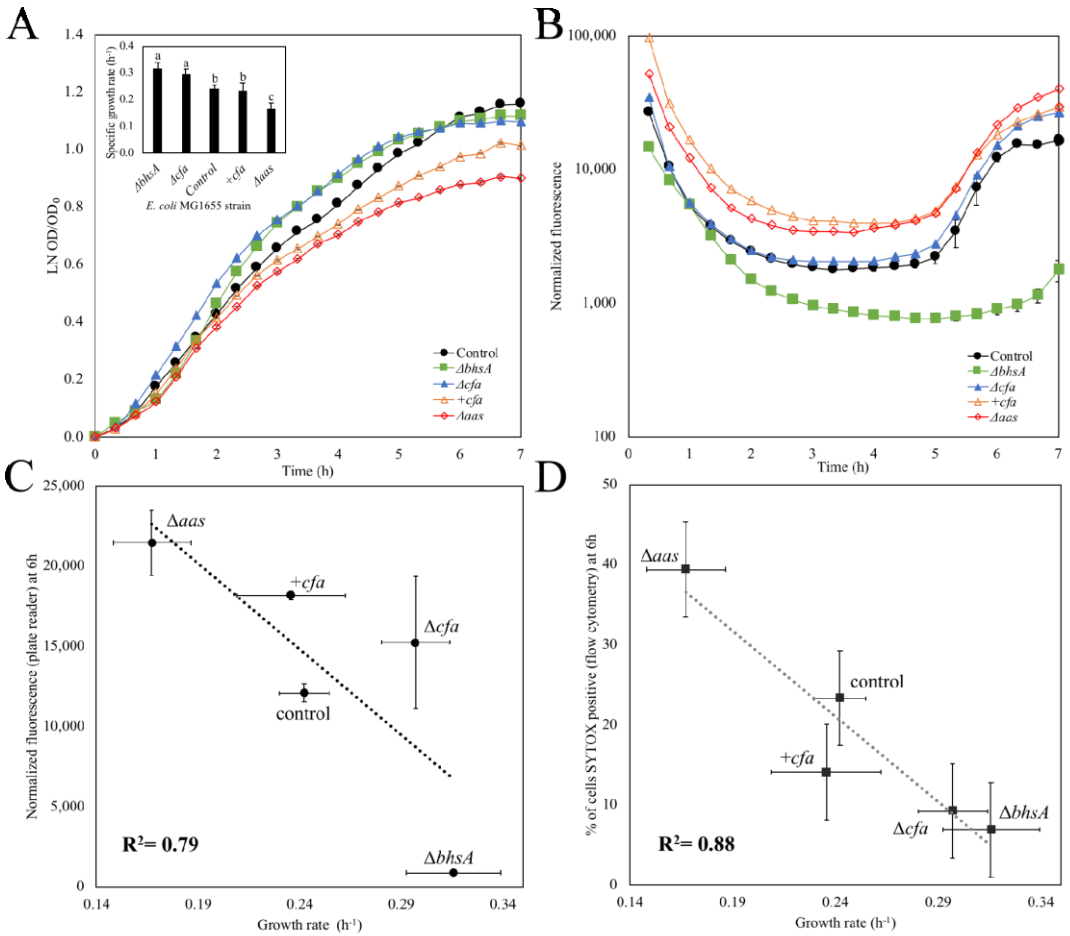


**Figure 4. *in situ* assessment of membrane engineering strategies to increase C8 tolerance**

Table 1. Strains utilized in this study.

| **Strains** | **Genotype** | **Reference** |
|---|---|---|
| MG1655 (WT) | F- lambda- *ilvG- rfb*-50 *rph*-1 | Jarboe's Lab |
| control | MG1655, Δ*ldhA*::FRT-*cat*-FRT, $Cm^+$ | [3] |
| +*cfa* | MG1655, pCA-*cfa*, $Amp^+$ | [4] |
| Δ*cfa* | MG1655, Δ*cfa*::FRT-*cat*-FRT, $Cm^+$ | [4] |
| Δ*bhsA* | MG1655, Δ*bhsA*::FRT-*cat*-FRT, $Cm^+$ | This study |
| Δ*aas* | MG1655, Δ*aas*::FRT-*cat*-FRT, $Cm^+$ | This study |

Table 2. Shared equipment user fees from five randomly selected US facilities.

| Facility | Flow cytometry cost ($/hr) | | Plate reader cost ($/hr) | |
|---|---|---|---|---|
| | internal user | external user | internal user | external user |
| 1 | 52 | 135 | 28 | 50 |
| 2 | 162 | 162 | 16 | 32 |
| 3 | 70 | 110 | 40 | 50 |
| 4 | 65 | 130 | 96 | 151 |
| 5 | 54 | 203 | 0 | 33 |
| Average cost | 81 | 148 | 36 | 63 |